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MUTAGENICITY OF PHOTOCHEMICALLY-TRANSFORMED POLYCYCLIC AROMATIC AMINES

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### ABSTRACT

Polycyclic aromatic amines (PAA) constitute a class of suspect genotoxic chemicals found in certain energy-related complex organic mixtures. In this report we review a variety of studies conducted in our laboratory over the past several years that concern the process of photochemical transformation (oxidation) of PAA and the increase in biological activity (genotoxicity) of the resulting complex mixtures of radiation-generated products. Biological endpoints of cytotoxicity and mutagenicity were measured in the Ames/Salmonella standard-plate assay. Typically, the assay was performed in the absence of metabolic enzymes (i.e., hepatic S9 fraction) unless a determination for promutagenic chemicals was required. Chemical fractionation and identification of various photoproducts was accomplished with h.p.l.c., UV and IR spectroscopic and mass spectrometric techniques. The benchmark PAA in our studies has been 2-aminofluorene (2-AF). We have shown that photooxidation of 2-AF can occur at both the exocyclic nitrogen and certain ring positions resulting in the formation of direct-acting and potent bacterial mutagens, including 2-nitrosofluorene, 2-nitrofluorene, and 2-nitrofluoren-9-one. In addition, we have observed that UVA-irradiated 2-AF solutions contain promutagenic compounds, i. e., photoproducts that can be bioconverted into ultimate mutagens by the addition of exogenous metabolic enzymes. An example is 2-aminofluoren-9-one. Our results to date support the hypothesis that the critical step in the activation of major, identified direct-acting mutagenic 2-AF photoproducts is their reduction by bacterial nitroreductase enzymes to reactive hydroxylamines. Photochemical oxidation is an alternative mechanism of transforming PAA into direct-acting genotoxins.

## INTRODUCTION

Primary aromatic amines (PAA) constitute a class of important industrial synthetic chemicals used as feedstocks in the production of various products, including dyes, pharmaceuticals, herbicides and plastics. They are also used in the rubber industry as antioxidants. However, PAA have recently been identified as a major contributor to the genotoxic properties of synthetic fossil fuels derived from coal or oil shale (Guerin et al., 1980; Tomkins and Ho, 1982; and Later et al., 1983). PAA are known potent promutagens/procarcinogens whose metabolism by microsomal mixed function oxidases and mechanism of action at the molecular level have been well documented (for reviews, see Miller, J.A., 1970; Miller, E.C., 1978; and Kriek and Westra, 1979). A critical step in the "activation" of a PAA to its ultimate mutagenic/carcinogenic form involves the oxidation of its exocyclic nitrogen(s) with the formation of an intermediate hydroxylamine that can yield a reactive, electrophilic nitrenium ion.

Aromatic nitro compounds, N-oxidized derivatives of PAA, are common constituents found in a variety of combustion effluents. They are potent and direct-acting microbial mutagens (Rosenkranz and Hermalstein, 1983) whose activities are dependent upon their reduction to hydroxylamines by a group of distinct nitroreductase enzymes (Rosenkranz et al., 1982). Certain nitroarenes have been shown to transform cultured mammalian cells (DiPaolo et al., 1983) and induce mammary gland tumors in rats (Hirose et al., 1984).

Thus it is apparent that the oxidation state of the exocyclic nitrogen substituents of PAA and aromatic nitro compounds is critical in the expression of genotoxicity of these two

classes of chemicals. In this report we review studies conducted in our laboratory (Okinaka et al., 1984a,b; Okinaka et al., 1985a,b; and Strniste et al., 1985a,b) concerning the process of photochemical transformation of PAA and the biological implications of such reactions. Our findings suggest that common environmental factors, including light and oxygen, can significantly modify the chemical composition of certain classes of pollutants during their residency time in the environment (i.e., from generation source to biological receptor of concern). We also elaborate on the pathway of light-mediated oxidative reactions that are responsible for the transformation of PAA into potent, direct-acting mutagens.

#### METHODS AND MATERIALS

Chemicals. 2-aminofluorene (2-AF, CAS registry no. 153-78-6) and the various 2-AF derivatives used as reference standards were obtained from either a commercial source (Aldrich Chemical Co., Milwaukee, WI) or were chemically synthesized. Solvents including acetonitrile, methanol and dimethyl sulfoxide (DMSO) were spectroscopic or HPLC grade.

Near Ultraviolet Light (UVA) Irradiation. Two parallel 15-W blacklights (GE F15T8 BLB) were positioned above the benchtop to yield a fluence of UVA (320-400 nm wavelengths) of 8.3 J/m<sup>2</sup>/s. Aliquots (4 ml) of a stock 5 mM solution of the desired chemical (in DMSO) were dispensed into individual 60 mm glass Petri dishes and covered with glass lids. The UVA irradiation was performed at room temperature for various exposure times (Strniste et al., 1985a). Irradiated samples were analyzed immediately or placed into amber-colored glass vials and stored at room temperature in the dark for future analysis.

**H.P.L.C. Analyses.** Analytical scale h.p.l.c. was performed as previously described (Strniste et al., 1985a). In summary, a 20  $\mu$ l aliquot of a desired sample was applied to a 10  $\mu$ m Radial-PAK C<sub>18</sub> cartridge (0.5 cm X 10 cm) fitted in a Compression Separation System (Z-Module, Waters Associates, Milford MA) and eluted with a programmed 30 min linear gradient of 1 mM triethylammonium bicarbonate buffer:acetonitrile (95:5 to 0:100) at a flow rate of 2 ml/min using a Beckman Model 334 Gradient Liquid Chromatograph System (Beckman Instruments, Inc., Fullerton, CA). This was followed by a 5 min wash of 100% acetonitrile. The eluate was continuously monitored for absorptivity at 254 nm. Reference standards of 2-AF and various 2-AF derivatives were analyzed separately, and their assignment to elution peaks based on their retention times ( $R_t$ ) are as noted in the figures. For semi-preparative scale h.p.l.c. analysis, a 250  $\mu$ l aliquot of a desired sample was applied to a replicate column and chromatographed as described above. The eluate was monitored for absorptivity at 254 nm and was generally fractionated into 10 or more samples of varying size.

**Cells.** Salmonella typhimurium tester strain TA1538 was obtained from Dr. Bruce Ames, University of California, Berkeley. Routine testing using established protocols was performed bimonthly to confirm the genotype of this strain (see Maron and Ames, 1983).

**Standard-Plate Assay.** The standard-plate assay, originally described by Ames et al. (1975), was performed without the addition of exogenous metabolic enzymes for the determination of direct-acting mutagenicity with minor modifications as previously reported (Strniste et al., 1985a). For the determination of promutagenic components in the irradiated 2-AF solutions, 15  $\mu$ l of rat liver homogenate (Aroclor-induced S9 mixture obtained from Litton Bionetics, Inc., Kensington, MD) was included in each reaction mixture according to the protocol

described by Maron and Ames (1983). Plates were incubated at 37° for 48 h before scoring for histidine positive (his<sup>+</sup>) revertant colonies. Assays were performed under subdued lighting, and each test sample was assayed at a minimum of three concentrations using triplicate plates per dose point. Tester strain authenticity was verified by its spontaneous reversion frequency to his<sup>+</sup> (historically for TA1538 we have obtained  $16 \pm 3$ ) and by reversion using appropriate reference mutagens. The solvent DMSO had no cytotoxic or mutagenic effects on this tester strain under the experimental protocols used in this report.

## RESULTS AND DISCUSSION

In previous reports we have shown that 2-AF solubilized in DMSO is photochemically transformed into a variety of oxidized derivatives after relatively short exposures to near ultraviolet light (UVA, radiation of wavelengths of 320-400 nm) (Okinaka et al., 1984a,b; Okinaka et al., 1985 a; and Strniste et al., 1985a,b). An example of this process is shown in Figure 1, which displays two analytical scale h.p.l.c. elution absorptivity profiles (eluate monitored at 254 nm) for unirradiated 2-AF and 2-AF exposed to 600 kJ/m<sup>2</sup> UVA. The UVA-irradiated 2-AF sample contains >30 discernible absorption peaks, five of which have been isolated and identified (Okinaka et al., 1984a,b; and Strniste et al., 1985a,b). Rapid photooxidation of 2-AF occurs primarily at the exocyclic nitrogen and at the carbon-9 position of the fluorene ring (Strniste et al., 1985a,b). Several of these oxidized derivatives, including 2-nitrosofluorene (2-NOF), 2-nitrofluorene (2-NO<sub>2</sub>F), and the unique 2-AF photoproduct, 2-nitrofluoren-9-one (2-NO<sub>2</sub>F-9-one), are direct-acting mutagens in

the Ames/Salmonella standard-plate assay.

There is a bimodal response in mutagenic potential of UVA-irradiated 2-AF solutions as a function of UVA exposure time (Strniste et al., 1985a). As is shown in the dose-response curves presented in Figure 2, the mutagenicity of selected UVA-irradiated 2-AF solutions on Salmonella tester strain TA1538 varies. There is a rapid rise in induced histidine ( $\text{his}^+$ ) revertants for solutions of 2-AF UVA irradiated from 0 to 150-200  $\text{kJ/m}^2$ . This increase in mutagenicity is followed by a decrease in photo-induced activity, which appears to be minimized at doses of UVA  $\sim 600\text{--}800 \text{ kJ/m}^2$ . A second rise in mutagenicity is seen that appears to peak at  $\sim 2000 \text{ kJ/m}^2$  UVA. Continued UVA exposure of irradiated solutions results in final abolition of mutagenic activity at a dose of  $\sim 4000 \text{ kJ/m}^2$ . It should be noted here that for natural sunlight there is a total dose of  $\sim 75 \text{ kJ/m}^2/\text{hr}$  UVA in the incident radiation received at the earth's surface.

In addition to UVA-induced, direct acting mutagenicity in solutions of 2-AF, we have also observed the phototransformation of 2-AF into promutagenic components; i.e., compounds that can be metabolized to ultimate mutagenic forms by the addition of exogenous rat hepatic S9 fraction. An example is 2-aminofluoren-9-one (2-AF-S-one), a photoproduct resulting from the rapid oxidation of 2-AF at the carbon-9 position of the fluorene ring. In Figure 3 we show an elution absorption profile from a semi-preparative scale h.p.l.c. of a  $600 \text{ kJ/m}^2$  UVA-irradiated 2-AF sample (panel A). Note the peak broadening due to the 12.5 times increase in the volume of sample injected. This volume was necessary for the mutagenicity assessment of fractionated samples of this same h.p.l.c. run (panel B). Eleven non-uniform fractions (4-10 ml) were collected in order to include specific absorption peaks in particular

fractions. Approximately 80% of the direct-acting mutagenicity recovered from this h.p.l.c. experiment is confined to the fraction collected between 20 and 23 min, which contains the elution peak of  $R_f$  of ~21 min. This particular elution peak contains both 2-NOF and 2-NO<sub>2</sub>F that are unresolvable under the described chromatograph conditions (Okinaka et al., 1984b; and Strniste et al., 1985a). About 10% of the direct-acting mutagenicity appears in the fraction collected between 18 and 20 min; this activity can be accounted for by the presence of a small amount of 2-NO<sub>2</sub>F-9-one, which is a major, direct-acting mutagenic species found in UVA-irradiated 2-AF solutions at longer exposure times (Strniste et al., 1985b). The remaining ~10% of direct-acting mutagenicity is spread throughout most of the other fractions and is chemically undefined to date.

As is shown in Figure 3 (panel B), about 95% of the promutagenic activity is partitioned into three major fractions (i.e., those collected between 13.5 and 15.5, 15.5 and 18, and 20 and 23 min) and the remaining ~5% of the activity is spread among three minor fractions (18-20, 23-25, and 25-30 min). Fractions of major activity are due to the presence of promutagenic 2-AF-9-one (fraction 13.5-15.5 min) and 2-AF (fraction 15.5-18 min). Fraction 20-23 min contains the direct-acting mutagens 2-NOF and 2-NO<sub>2</sub>F that are not dependent on exogenous metabolic enzymes for expression of their activity. Therefore, this peak of "promutagenic activity" can be accounted for by the presence of these two direct-acting mutagens. A similar argument can be given for the 18-20 min fraction of minor activity that contains small amounts of the direct-acting mutagen 2-NO<sub>2</sub>F-9-one. The identity of the compounds responsible for the promutagenic activity in the other two minor fractions is unknown at this time.

In Figure 4 we compare direct-acting mutagenicity to promutagenic activity of irradiated 2-AF solutions exposed to varying UVA doses. As mentioned above and discussed elsewhere (Strniste et al., 1985a), there is a bimodal response in the direct-acting mutagenic potential of UVA-irradiated 2-AF solutions as a function of radiation dose. This phenomenon, also depicted in Figure 4 (upper panel), has been interpreted as follows: a) the rapid formation of mutagenic 2-NOF that maximizes in amount between 150-200 kJ/m<sup>2</sup> UVA; b) a subsequent reduction in mutagenicity due to the loss of photolabile 2-NOF; c) a new increase in mutagenicity due to the formation of 2-NO<sub>2</sub>F that maximizes in amount at ~1800 kJ/m<sup>2</sup> UVA; d) a continued increase in mutagenicity due to the formation of 2-NO<sub>2</sub>F-9-one, a later appearing mutagenic photoproduct of UVA-irradiated 2-AF-9-one; and e) complete photodestruction of direct-acting mutagenic 2-AF derivatives at UVA doses of ~4000 kJ/m<sup>2</sup> or greater.

The bottom panel of Figure 4 displays the promutagenic activity of the same series of UVA-irradiated 2-AF solutions (i.e., mutagenicity determined with the addition of exogenous rat hepatic S9 fraction). 2-AF itself is a potent promutagen. Exposure of 2-AF to UVA results in its chemical transformation into various photoproducts, some of which are direct-acting mutagens, others are promutagenic, and still others are non-mutagenic. The decay of activity seen with increasing UVA doses is associated with the phototransformation of the two primary promutagens, i.e., parental 2-AF itself, which is absent in the irradiated solution at UVA doses >900 kJ/m<sup>2</sup>, and the early photoproduct 2-AF-9-one, which is completely phototransformed at UVA doses >1800 kJ/m<sup>2</sup>. At greater UVA doses (e.g., 2000 kJ/m<sup>2</sup> UVA), a substantial amount of mutagenicity of the irradiated 2-AF solution can be attributed to the presence of the direct-acting mutagens 2-NO<sub>2</sub>F and

2-NO<sub>2</sub>F-9-one and other undefined promutagenic species. Extensive UVA irradiation destroys the promutagenicity of 2-AF solutions.

In Figure 5 we present an up-to-date schematic depicting the photooxidation of 2-AF. Oxidation occurs rapidly at either the exocyclic nitrogen, resulting in the formation of direct-acting mutagenic 2-NOF and 2-NO<sub>2</sub>F, or at the carbon-9 of the fluorene, ring resulting in the formation of promutagenic 2-AF-9-one. We have not been able to detect hydroxylamine species in the irradiated 2-AF solutions, possibly due to their instability in DMSO or to their irreversible binding to the C<sub>18</sub> resin used in the h.p.l.c experiments (unpublished observations). However, indirect evidence for its presence in the irradiated 2-AF solutions comes from the identification of the photoproduct 2,2'-azoxy *bis*fluorene, which is the result of the condensation of the hydroxylamine with 2-NOF (Coombes, 1979). The continued increase of 2-NO<sub>2</sub>F in an irradiated solution even after the total phototransformation of parental 2-AF implies that it can arise via an additional pathway(s) that is unknown at this time. The formation of the unique, direct-acting mutagenic 2-NO<sub>2</sub>F-9-one is a consequence of further photooxidation of 2-AF-9-one. However, to date we have not been able to detect the presence of the suspect intermediates, 2-(hydroxyamino)fluoren-9-one or 2-nitrosofluoren-9-one. Conversion of 2-NO<sub>2</sub>F to 2-NO<sub>2</sub>F-9-one by oxidation at the carbon-9 position of the fluorene ring does not appear to be a significant pathway. (Strniste et al., 1985b). Ring hydroxylation of 2-NO<sub>2</sub>F and 2-NO<sub>2</sub>F-9-one is also a conceivable photochemical reaction. This possibility is based on the following observations: 1) recent experimental findings reported by Yasuhara and Fuwa (1983) and Lofroth et al. (1984) demonstrating light-induced formation of 1-nitropyren-2-ol

in irradiated solutions of 1-nitropyrene; and 2) preliminary unpublished results from our laboratory in which we have detected suspect phenolic species in certain h.p.l.c. fractions using a sensitive, colorimetric assay (Tsen, 1961; and Frederick et al., 1981).

Of the >30 photoproducts formed in UVA-irradiated solutions of 2-AF, we have positively identified five and speculate on the presence of at least six other compounds. Further chemical fractionation and identification will be required to totally assess the complex mixture of products generated in the photolysis of 2-AF

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#### FIGURE LEGENDS

FIGURE 1. Analytical scale h.p.l.c. experiment for unirradiated 2-AF (panel A) and 600 kJ/m<sup>2</sup> UVA-irradiated 2-AF (panel B). Aliquots (20 µl) of unirradiated or irradiated 2-AF were chromatographed as described in the Methods and Materials section and the eluate monitored continuously at 254 nm. Designated reference standards (commercially available or chemically synthesized) were run separately and their elution positions are as noted.

FIGURE 2. Direct-acting mutagenicity of UVA-irradiated 2-AF in Salmonella TA1538.

Aliquots (4 ml) of a stock 5 mM 2-AF solution (in DMSO) were exposed to UVA for various doses (0-4000 kJ/m<sup>2</sup>) and tested for mutagenicity in the Ames/Salmonella standard-plate assay without the addition of exogenous rat hepatic S9 mixture. Numbers above each dose-response curve represent total UVA dose individual 2-AF solutions received. Arrow bars represent  $\pm 1$  S.D.

FIGURE 3. Semi-preparative scale h.p.l.c. experiment of 600 kJ/m<sup>2</sup> UVA-irradiated 2-AF (panel A) and the direct-acting mutagenic (-S9) or promutagenic (+S9) activity of the fractionated eluate on Salmonella TA1538 (panel B).

FIGURE 4. Comparison of direct-acting mutagenicity (-S9) to the promutagenicity (+S9) of various UVA-irradiated 2-AF solutions when assessed on Salmonella TA1538. Aliquots (4 ml) of a stock 5 mM 2-AF solution (in DMSO) were exposed to varying UVA doses (0-4000 kJ/m<sup>2</sup>) and assayed in the Ames/Salmonella standard-plate assay with or without the addition of exogenous rat hepatic S9 mixture.

FIGURE 5. Schematic of the photooxidation of 2-AF. The various chemical structures are:  
 1) 2-aminofluorene; 2) 2-(hydroxyamino)fluorene; 3) 2-nitrosofluorene; 4) 2-nitrofluorene;  
 5) 2-aminofluoren-9-one; 6) 2-(hydroxyamino)fluoren-9-one; 7) 2-nitrosofluoren-9-one;  
 8) 2-nitrofluor-9-one; 9) 2,2'-azoxy *bis*fluorene; 10) 2,2'-azoxy *bis*fluoren-9-one;  
 11) hydroxy-2-nitrofluorene; and 12) hydroxy-2-nitrofluoren-9-one.









